Proc. 25, 519.
Niyogi, S. K., and Stevens, A. (1965), *J. Biol. Chem. 240*, 2593.
Pitha, P. M., Huang, W. M., and Ts'o, P. O. P. (1968), *Proc. Natl. Acad. Sci. U. S. 61*, 332.
Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., and Hollis, D. P. (1968), *J. Am. Chem. Soc. 90*, 1042.
Schweizer, M. P., Chan, S. I., and Ts'o, P. O. P. (1965), *J. Am. Chem. Soc. 87*, 5241.
Steck, T. L., Caicuts, M. J., and Wilson, R. G. (1968), *J. Biol. Chem. 243*, 2769.
Stevens, C. L., and Felsenfeld, G. (1964), *Biopolymers 2*, 293.
Straat, P. A., and Ts'o, P. O. P. (1969), *Fed. Proc. 28*, No. 2, 9000.
Straat, P. A., Ts'o, P. O. P., and Bollum, F. J. (1969), *J. Biol. Chem. 244*, 391.
Tanford, C. (1961), *in* Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 526.
Ts'o, P. O. P., and Chan, S. I. (1964), *J. Am. Chem. Soc. 86*, 4176.
Ts'o, P. O. P., and Huang, W. M. (1968), *Biochemistry 7*, 2954.
Ts'o, P. O. P., Melvin, I. S., and Olson, A. C. (1963), *J. Am. Chem. Soc. 85*, 1289.
Ts'o, P. O. P., and Schweizer, M. P. (1968), *Biochemistry 7*, 2963.
Uhlenbeck, O., Harrison, R., and Doty, P. (1968), *in* Molecular Association in Biology, Pullman, B., Ed., New York, N. Y., Academic, p 107.

Further Studies of the Molecular Weight in Aqueous Solution of the Low-Sulfur Wool Protein Component 8 and a Reinterpretation of Previous Results*

P. D. Jeffrey

ABSTRACT: In a previous sedimentation equilibrium study in 0.01 M sodium tetraborate it was found that at low concentration the measured molecular weight of the reduced and alkylated low-sulfur wool protein, component 8, increased with increasing protein concentration. The variation of molecular weight was satisfactorily explained as the consequence of a rapid reversible equilibrium between a monomer of mol wt 23,000, dimer, and trimer. The present paper gives the results of frontal analysis gel filtration on Sephadex G-200 and osmotic pressure experiments which show that component 8 is almost certainly *not* involved in such an equilibrium in 0.01 M sodium borate. These results together with those from more high-speed sedimentation equilibrium experiments and the use of two-species plots indicate that the component 8 used in these studies is a mixture of more than 95% of a protein of mol wt 45,000 and less than 5% of one of mol wt 14,000. Calculations using these figures show that the results of the high-speed sedimentation equilibrium experiments can indeed be explained on this basis and it is concluded that the molecular weight of component 8 is 45,000.

In a previous paper (Jeffrey, 1968) I presented the results of a sedimentation equilibrium study in 0.01 M sodium tetraborate of the reduced and alkylated low-sulfur wool protein termed component 8. The molecular weight *vs.* concentration data were explained in terms of thermodynamic nonideality and a rapid, reversible equilibrium between a monomer of mol wt 23,000 and the dimer and trimer of this unit. The present paper gives the results of an extensive investigation of component 8 in the same solvent by gel filtration, sedimentation equilibrium and osmometry, and shows that the protein is almost certainly not involved in such an equilibrium and that the results presented in the previous paper can be explained in a different way.

Gel filtration on Sephadex was used both as a method of fractionation and as a technique for examining an interacting system by the method of frontal analysis. It was the latter application which originally indicated that component 8 was not an associating system in sodium tetraborate solution and made it necessary to carry out more sedimentation equilibrium experiments and to reinterpret the results obtained before. A determination of the molecular weight of component 8 by osmotic pressure also reported in this paper provides additional support for the explanation of the high-speed sedimentation equilibrium results advanced here.

Materials

Buffer. The buffer salt was analytical reagent grade sodium tetraborate and 0.01 M solutions were made with glass-distilled water. The pH was measured and if necessary adjusted to pH 9.20.

Component 8. The component 8 used in the experiments reported here came from three different preparations. In all of these the component 8 was fractionated from the soluble low-

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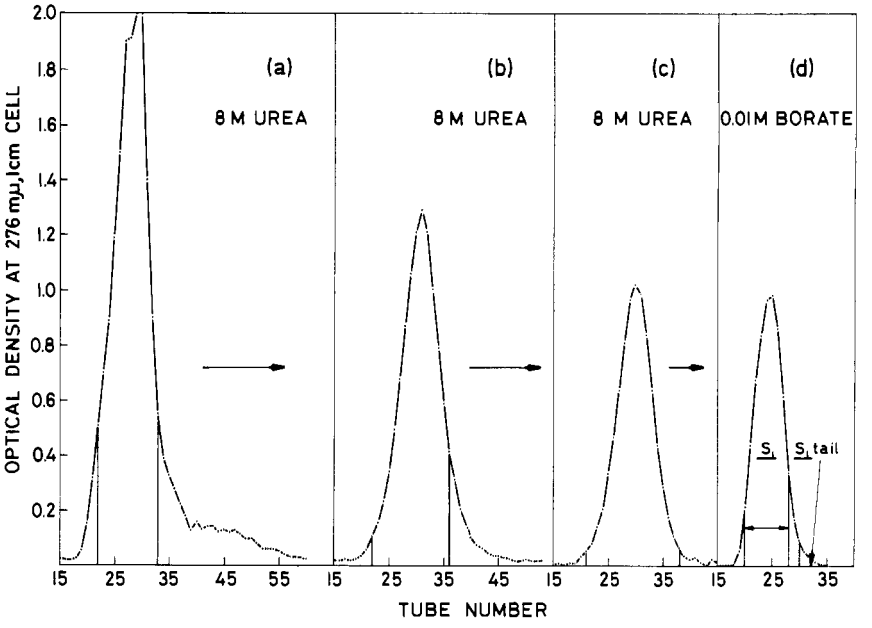

FIGURE 1: Gel filtration of component 8 on Sephadex G-200. The experimental details are given in the text.

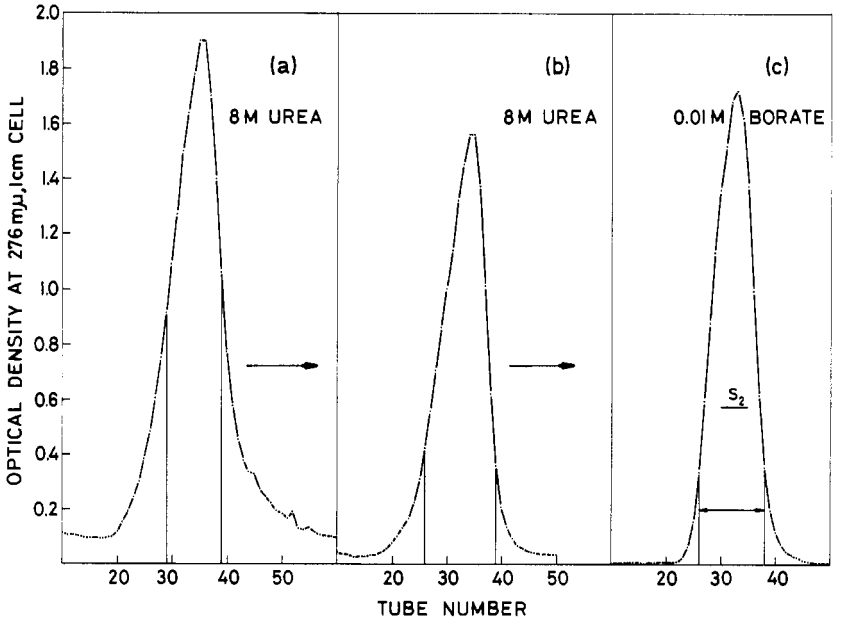

FIGURE 2: Gel filtration of component 8 on Sephadex G-200. The experimental details which are the same as those for Figure 1 are given in the text.

sulfur proteins obtained by reduction and S carboxymethylation of merino wool by the acetone precipitation method of Dowling and Crewther (L. M. Dowling and W. G. Crewther, unpublished data) and different preparations differ only in the number of times they were fractionated on Sephadex G-200 subsequent to the original extraction. The gel filtration behaviour of five different preparations of component 8 was examined fairly thoroughly (see under Methods) and was reproducible in its qualitative aspects from one preparation to the next. Briefly, it was found that when a sample of component 8 was applied to Sephadex G-200 after extraction it gave an elution profile consisting of a main peak, apparently some aggregated material, and a broad trailing edge (Figures 1 and 2). The amount of aggregate and tail material varied slightly from preparation to preparation but three or four passages through the column were always required to reduce the size of the tail to an acceptable level (Figures 1 and 2). The samples called A and S used in the study reported previously (Jeffrey, 1968) would correspond to Figure 1a and the cut taken from Figure, 1a respectively (or Figure 2a and the cut taken from 2a). The samples used here which should be essentially identical are denoted S_1 and S_2 and are the cuts shown in Figures 1d

and 2c, respectively. The frontal analysis and the sedimentation equilibrium runs were done with S_1; the osmometry was done with S_2. Both samples of component 8 were stored in the refrigerator in solution in 0.01 M sodium tetraborate.

Other Proteins. Reduced and alkylated feather keratin was prepared from duck feather rachis by the method of Harrap and Woods (1964) and was stored in solution in 0.01 M sodium tetraborate in the refrigerator. The ovalbumin was Pentex, five-times-crystallized lot 5.

Methods

Gel Filtration. FRACTIONATION. The fractionation of component 8 was done at room temperature (20 ± 1°) on 130 × 2.3 cm columns of sieved Sephadex G-200 equilibrated with either 0.01 M sodium tetraborate (pH 9.20) or with solvent 8 M with respect to urea consisting of 0.01 M Tris and 0.001 M EDTA and pH 10.1. Between 100 and 250 mg of protein dissolved in 10–20 ml of buffer were applied carefully to the top of the bed and washed in with two 10-ml aliquots of buffer. The column was then connected to a pump and solvent was pumped through at the rate of 15 ml/hr. Fractions were collected at 30-min intervals and their optical densities were measured in a 1-cm cell at 276 mμ. After the elution profile was obtained and the required cut made, the protein solution was dialyzed free of urea or borate, freeze dried, and redissolved in a suitable volume of solvent for reapplication to a column. The protein solutions were filtered through 1.2-μ Millipore filters before application to the column and the urea buffer was passed through a mixed-bed ion-exchange resin to remove cyanate ions. Five different preparations of component 8 were applied to G-200 columns in the course of this work either in 8 M urea several times followed by 0.01 M sodium tetraborate or *vice versa*. In some cases material from the tail and the main peak after the first passage through the column was taken and used for amino analyses. The information obtained from this gel filtration will not be discussed in detail because it did not lead to any quantitative conclusions; however a few general remarks can be made. (1) Preparations of component 8 put onto columns usually show the presence of some aggregate and always have a tail which varies in amount. (2) There was no evidence of increased aggregation of the samples in the absence of urea. (3) It took at least three and sometimes four passages through such columns to remove visible evidence of a significant tail and although the 8 M urea solution originally produced a larger amount of tail material than did the 0.01 M sodium borate there was some evidence that the borate removed at least some material that was different from that removed by urea. For this reason at least one passage through a sodium borate column was included in the preparation of a sample for further work. (4) The amino acid analyses were inconclusive as samples from the tails were obviously contaminated with material from the peak. Such trends as were noticeable suggested that the tail material could be mainly high glycine–tyrosine proteins together with high sulfur proteins (Crewther *et al.*, 1965).

FRONTAL ANALYSIS gel filtration is carried out by loading sufficient protein solution onto a small column of Sephadex that the concentration of the effluent reaches a plateau equal in concentration to the solution being loaded. It is a useful technique for studying associating or suspected associating protein systems as one can examine the boundary shapes and the dependence of elution volumes upon concentration down to very low concentrations and also compare the shapes of ascending (solution–solvent) and descending (solvent–solution) boundaries as in electrophoresis. Thus one can tell whether or not an interaction is occurring and, if so, often get additional information about the sort of equilibrium that is present (Winzor and Scheraga, 1963). For the present work I used a freshly packed 29 × 0.43 cm column of sieved Sephadex G-200 equilibrated with 0.01 M sodium tetraborate of pH 9.20. A protein solution of the required concentrations was prepared by dilution of a stock solution and the concentration checked by measuring the optical density at 220 mμ in a 2-mm quartz cell. The solvent level in the column was allowed to fall until the top of the bed was just exposed and 15 ml of protein solution were immediately loaded from a pipet. Effluent was collected in weighed tubes from the time of commencement of loading. The use of drop counting allowed fractions of uniform size (about 0.5 ml) to be collected; the flow rate was adjusted to be constant and close to 5 ml/hr by using a Marriott tube and adjusting the height of the flexible outlet tubing. When the last of the protein solution had flowed into the column and the top of the bed was just exposed, solvent was loaded and effluent collection was continued until the original base line was regained. The tubes were weighed and the volume of each fraction was calculated (assuming a density of 1.0 g/ml) and its optical density measured at 220 mμ in a 2-mm path-length quartz cell. A 2-mm cell was used because the volume of each fraction was too small to use a larger cell and the wavelength 220 mμ was chosen as the lowest one (nearest to the peptide bond absorption maximum) at which the spectrophotometer (Beckman DU) would measure satisfactorily. Sodium tetraborate solutions do not absorb at this wavelength and its use gave an order of magnitude increase over those at 276 mμ of the optical densities of the protein-containing fractions.

OSMOMETRY. The osmotic pressure measurements were made at 25° with a Model 501 Mechrolab high-speed Servo osmometer and UFF superdense grade Sartorius Membran filters. All the parts of the instrument were thoroughly cleaned with hot concentrated detergent and rinsed with distilled water before each experiment. Solvents were degassed and filtered through 0.45-μ Millipore filters before use. Protein solutions were filtered in the same way. The membranes were conditioned by soaking overnight in the cold in the solvent to be used and were degassed in warm solvent under vacuum from a water faucet aspirator before use. The solvent contained 0.005% of the detergent Tween 80 to offset the surface tension of the aqueous solutions and the stock protein solution was dialyzed against solvent before an experiment. A bubble size of $^3/_{16}$ in. was found to be satisfactory and the instrument was always operated in reverse mode so that the bubble would not be lost under the membrane. The reading with solvent on either side of the membrane was established each day although not necessarily between each protein solution determination. A reading for a protein solution was judged to be satisfactory if it varied by less than 0.01 cm in 10 min and if it could be repeated within ±0.03 cm.

The molecular weights of ovalbumin and of reduced and alkylated feather keratin were measured as a check on the technique and the instrument. The ovalbumin was dissolved in sodium phosphate–sodium chloride buffer of ionic strength 0.1 and pH 7.0 and the feather keratin in 0.01 M sodium tetra-

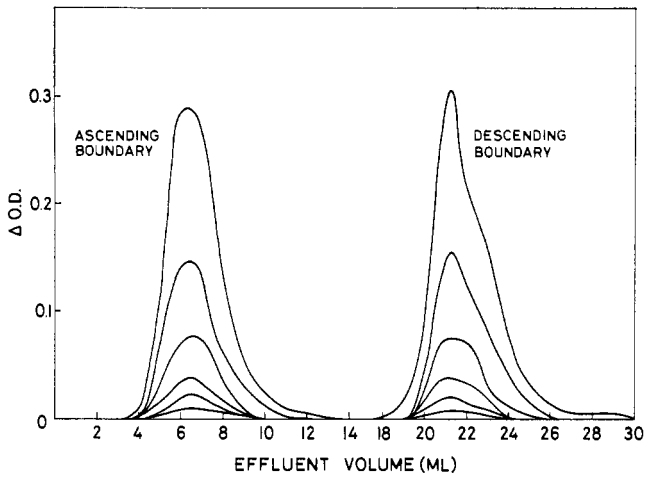

FIGURE 3: First derivative curves from frontal analysis of component 8 on Sephadex G-200 in 0.01 M sodium borate. The concentrations of the six solutions are given in the text; the highest was just under 0.1 g/100 ml. Note that in this diagram some of the plateau region has been cut out.

borate–0.1 M sodium chloride of pH 9.2. The molecular weight obtained for ovalbumin was 46,300 which is in good agreement with a meniscus depletion–sedimentation equilibrium run on the same sample which gave 46,000. Both values are in satisfactory agreement with those in the literature (Tanford, 1963). The value obtained for reduced and alkylated feather keratin was 10,400 which is in excellent agreement with that of 11,000 measured by sedimentation equilibrium by Harrap and Woods (1964).

The concentrations of ovalbumin and reduced and alkylated feather keratin stock solutions of concentration about 1 g/100 ml were determined by a semimicro Kjeldahl technique. Dilutions for use in the osmotic pressure experiments were accurately made from these stock solutions.

An extinction coefficient was measured for component 8 in 0.01 M sodium tetraborate at 278 mμ from semimicro Kjeldahl determinations on two 1-ml samples of concentration about 1 g/100 ml and a per cent nitrogen of 16.7 (measured for SCMKA[1] by Harrap and Woods, 1958), giving $E^{1\%}_{1\,cm}$ 7.06. The concentration in grams per liter of each dilution used in the osmotic pressure experiment was calculated from this figure and the optical density at 278 mμ of the solution was read immediately before its introduction to the osmometer.

Sedimentation Equilibrium Experiments. The experiments were done exactly as described in preceding papers (Jeffrey, 1968). All of the new experiments described were meniscus depletion experiments and photographs at sedimentation equilibrium were taken using the Rayleigh interference optical system. Apparent weight-average molecular weights were evaluated from the expression

$$M_{w\mathrm{app}(r)} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2}\left(\frac{\mathrm{d}\ln j}{\mathrm{d}(r^2)}\right)_r \qquad (1)$$

where R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solvent, ω is the angular velocity, and j is the protein concentration in terms of Rayleigh interference fringes in the 12-mm ultracentrifuge cell. (d ln j/d(r^2))$_r$ was evaluated from eq 13 of Yphantis (1964). Number-average σ_n ($\sigma = \omega^2 M \cdot (1 - \bar{v}\rho)/RT$ of Yphantis (1964)) were evaluated from

$$\sigma_n(r_k) \simeq \frac{j(r_k)}{I + \dfrac{\Delta r}{2m}\sum_{i=2}^{k} r_i j(r_i) + r_{i-1} j(r_{i-1})} \qquad (2)$$

where $I = j(r_1)/\sigma_\omega(r_1)$ and m is the horizontal magnification factor of the ultracentrifuge optical system (Yphantis, 1964). r_1 was taken as the point at which the fringe displacement was 100 μ. These $\sigma_{n's}$ were converted into point number-average molecular weights by multiplying by the constant $RT/\omega^2 \cdot (1 - \bar{v}\rho)$. The value used for $\bar{v}$ for component 8 was 0.715 ml/g and was measured in accordance with the Casassa and Eisenberg (1964) definition of macromolecular component. The use of this value and the possible magnitude of charge effects with component 8 in 0.01 M sodium tetraborate at pH 9.2 are discussed in a previous paper (Jeffrey, 1968). It was concluded that any errors in the extrapolated value for the minimum molecular weight due to the charge on the protein would be no greater than experimental error.

Results

Frontal Analysis. Figure 3 shows the first derivative curves obtained from frontal analysis gel filtration with 6 different concentrations of component 8. The component 8 used in these experiments was that from the cut S1 shown in Figure 1d and the initial concentrations were (in g/100 ml) 0.097, 0.049, 0.024, 0.012, 0.006, and 0.003. The concentration range over which it is profitable to measure the elution volume of a suspected rapidly polymerizing system depends upon the equilibrium constant or constants governing the reactions. These constants determine the proportions of polymers coexisting at a given concentration and thus the steepness of the weight-average elution volume *vs.* concentration curve. In the case of the present system it is known from the sedimentation equilibrium results that the weight-average molecular weight *vs.* concentration curve only begins to turn down at concentrations below 0.1 g/100 ml and this trend only becomes marked at concentrations below about 0.025 g/100 ml. That is, if the change in weight-average molecular weight with concentration *is* caused by chemical reaction, the equilibrium constant (or constants) are of such magnitude as to give rise to a distribution of polymeric species which affects the weight-average molecular weight to a detectable extent only below a concentration at the most of 0.1 g/100 ml. The same will be true of the weight-average elution volume which as Winzor and Scheraga (1964) show is inversely proportional to the weight-average molecular weight in such systems. Since the weight-average molecular weight *vs.* concentration curve is either horizontal or decreases in the opposite direction due to thermodynamic nonideality at protein concentrations above 0.1 g/100 ml, it must be concluded that if an equilibrium is present it is so far over to the right at this concentration that there is no point in measuring elution volumes at higher concentrations because the only variation with concentration they will show is that

[1] SCMKA is the reduced and alkylated low-sulfur protein extract from wool of which component 8 is a major fraction.

due to nonideal elution behavior analogous to that shown at the higher concentrations in the sedimentation equilibrium experiments (Winzor and Scheraga, 1964). The first derivatives of the ascending and descending boundaries were obtained by drawing smooth curves through the measured optical density *vs.* volume plots and taking first differences between values read off at equal 0.5-ml volume increments. The maximum of the resulting peak which corresponds to the inflection point of the integral curve gives the elution volume corresponding to the plateau concentration of protein, *i.e.*, to the initial concentration. A plot of the elution volume of the component 8 ascending boundary against initial concentration is given in Figure 4. The mean of the six elution volumes for the ascending boundary was 6.44 ± 0.08 ml while that for the descending boundary was 21.25 ± 0.06. The error here is of the same order as the volume of a single drop (about 0.05 ml) and it was therefore concluded that the elution volumes of both ascending and descending boundaries did not vary with concentration. Another way of analyzing the results of frontal analysis gel filtration experiments on suspected interacting systems has recently been proposed by Ronalds and Winzor (1969). The quantity $(c_a + c_t - c_0)$ is evaluated as a function of the volume of effluent, where c_a is the concentration in the advancing profile, c_t is the concentration at the same volume (measured with respect to V_e, the appropriate elution volume) in the trailing profile, and c_0 is the initial protein concentration, *i.e.*, the plateau concentration.

The integral

$$\int_0^{V'} (c_a + c_t - c_0)_v dV = 0$$

where V' is a point on the base line beyond the trailing profile, is a statement of the conservation of mass and Ronalds and Winzor denote the area given by the integral from 0 to V_e by A_1 and that from V_e to V' by A_2. For a solute such as bovine plasma albumin exhibiting concentration dependence of the type, $V_e = V_e^0 + kc_0$, where V_e^0 is the elution volume at infinite dilution, Ronalds and Winzor show that $A_1 = kc_0^2/4$ and that A_1 is positive and A_2 negative, whereas for an interacting system where the concentration dependence is not simply related to A_1, A_1 is negative and A_2 positive. A finite value for these areas shows that the advancing and trailing boundaries are nonenantiographic, *i.e.*, are not mirror images of each other. The considerations outlined above provide a method for detecting nonenantiography, measuring its magnitude and deciding whether it is due to positive concentration dependence or to chemical interaction, without having to differentiate manually the concentration *vs.* volume data obtained experimentally, a process which increases experimental uncertainty.

I have applied this technique to the experimental results obtained with all six concentrations of component 8 and in each case obtained the result $A_1 = A_2 = 0$, that is to say there is no concentration dependence of the elution volume of component 8 in this concentration range. This is in agreement with the conclusion reached after simply plotting the elution volumes *vs.* the concentrations (Figure 4). A previous interpretation of the results of high-speed sedimentation equilibrium experiments with this system in terms of a rapid equilibrium between a monomer (of molecular weight 23,000), dimer, and trimer

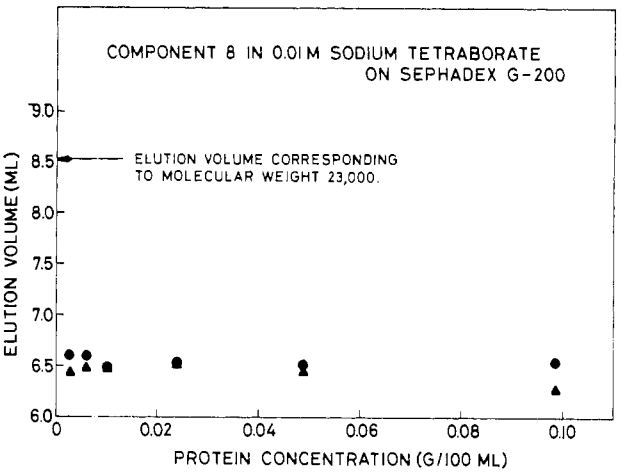

FIGURE 4: Variation of elution volume of component 8 with concentration. The points refer to the ascending boundary. (▲) Peak maximum from first derivative curve, (●) point of inflexion of integral curve.

(Jeffrey, 1968) would lead one to expect an increase in elution volume with decreasing concentration (Winzor and Scheraga, 1963). To check that the appropriate increase in elution volume was in fact detectable on the column used here, a solution of reduced and alkylated feather keratin (mol wt 10,500) of concentration 0.08 g/100 ml was put through the column. The elution volume of the ascending boundary was 11.12 ml. Now we have for the ascending boundary component 8 with a molecular weight of 45,000 at a concentration of 0.1 g/100 ml from the low-speed sedimentation equilibrium experiments where no appreciable fractionation would have occurred, and an elution volume at the same concentration of 6.44 ml, and reduced and alkylated feather keratin mol wt 10,500 and elution volume 11.12 ml. If it is assumed that the relationship between the logarithm of the molecular weight and the elution volume is linear then the two pairs of values quoted above define the linear relationship for the column used between the mol wt 10,500 and 45,000. Thompson and O'Donnell (1965) have shown that a linear relationship is obeyed for reduced and carboxymethylated proteins in 8 M urea so we may assume that such a relationship is generally obeyed by reduced and alkylated proteins in the random coil conformation. Reduced and alkylated feather keratin is in this form in 0.01 M sodium tetraborate. Component 8 however has about 50% helix content in aqueous solution (Thompson and O'Donnell, 1965) so its elution behavior may not be strictly comparable with reduced and alkylated feather keratin. Unfortunately there are no proteins with the necessary requirement of about 50% helix content in aqueous solution in the reduced and alkylated form available for calibrating the column and feather keratin was the nearest approximation to a homologous protein which could be found for this purpose. For this reason and also because only two values were used to establish the line the procedure is a rather crude approximation. All we are trying to show here though is that the order of magnitude of the difference in elution volumes for a variation in the protein molecular weight by a factor of two, on this column, is significantly greater than the experimental error of measuring elution volumes. The value obtained for the elution volume at infinite di-

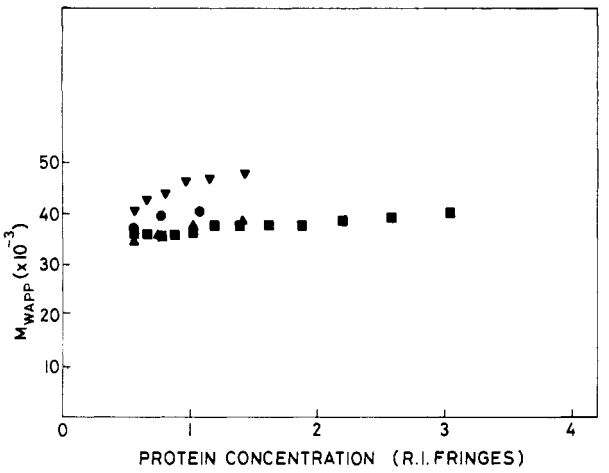

FIGURE 5: Meniscus depletion experiments with the peak tube of sample S1 of component 8 in 0.01 M sodium borate (pH 9.2) at 25°, 3-mm columns. (●) 0.14% 44,770 rpm. (▲) 0.14% 44,770 rpm 6 weeks after (●). (▼) 0.014% 44,770 rpm. (■) 0.14% 31,410 rpm.

lution for a protein of mol wt 23,000 was 8.52 ml and is shown in Figure 4. The actual value here is not important but the fact that a change in the molecular weight by a factor of 2 produces a change in the elution volume about 40 times as great as the experimental error in measuring elution volumes (0.05 ml) means that the experiments would easily detect a dissociation to units of mol wt 23,000 if it occurred. It can also be noted that Winzor and Scheraga (1964) in experiments with α-chymotrypsin on a column of Sephadex 32 × 1.25 cm found a difference of 2.36 ml in elution volumes for molecular weights of 24,000 and 40,000. The order of magnitude of this change is in agreement with that found above. It therefore has to be concluded on the evidence of the frontal analysis that component 8 does not dissociate to units of mol wt 23,000. The only way in which invariance of the weight-average elution volume with concentration could be obtained in a dissociating system would be for a conformational change on dissociation to compensate for the increase in elution volume produced by the presence of species of low molecular weight. This seems very unlikely. Since all of the concentrations examined were below 0.1 g/100 ml an increase in elution volume with increasing concentration of the type normally encountered when proteins are eluted from Sephadex columns (Winzor and Nichol, 1965) was not expected.

A comparison of the shapes of the ascending and descending boundaries is also instructive in deciding whether a protein is associating and what species are present. In an associating system it is expected that the ascending boundary becomes increasingly sharpened with respect to the descending boundary as the concentration is lowered. This is not the case with the present experiments, as if it were, A_1 would be negative and A_2 positive (see above and Ronalds and Winzor, 1969). The analysis of the sedimentation equilibrium experiments was consistent with the hypothesis that component 8 in 0.01 M sodium tetraborate consisted of a monomer in equilibrium with dimer and trimer, with the trimer being highly favored over the dimer. If such a system is considered to be essentially a monomer–trimer system, the descending boundary (corresponding to the boundary in a sedimentation velocity experiment) would be expected to show partial resolution into two peaks. At the lowest concentration the slower of these would be present alone. As the concentration is raised a faster peak should appear and grow in size and velocity while the size and position of the slower one remains constant (Winzor and Scheraga, 1963). This behavior is not demonstrated by component 8. In the case of a monomer–dimer–trimer system such partial resolution does not necessarily occur; however in this case the weight-average elution volume (or sedimentation coefficient) of the descending boundary will show concentration dependence. It has been shown that this behavior is not demonstrated by component 8. There is a shoulder or tail on the descending boundary and a trace of this on the ascending boundary but this seems to be just low molecular weight material which even four passages (Figure 1) through Sephadex have not quite removed. The fact that the tail is shown more clearly on the descending boundary is probably due to the smoothing procedure before differentiation together with the beginning of the plateau in the corresponding region of the ascending boundary. The results of the analysis of the ascending and descending boundaries by the method of Ronalds and Winzor (1969) are in agreement with this explanation as they show that the ascending and descending boundaries are enantiographic. The frontal analysis therefore seems to show that component 8 is not an associating system and that the protein sample which has been called component 8 is most likely largely a protein of a single molecular weight together with some material of lower molecular weight.

Sedimentation Equilibrium. Figure 5 shows the results of four meniscus depletion experiments with component 8 from the peak tube (tube 25) of S1 (Figure 1). The molecular weights increase with concentration in the way previously found with this protein (Jeffrey, 1968) but the fact that the tenfold dilution produced a marked increase in the measured molecular weights shows that the dependence upon concentration is partly or wholly due to their being a mixture of proteins present. No systematic dependence upon speed or concentrations was found previously and it was concluded that the small variation in the molecular weights obtained from different experiments was due to experimental errors. The two experiments on the same solution at an interval of 6 weeks shown in Figure 5 were done to try to establish whether a slow dissociation or aggregation might be occurring. It is apparent that no aggregation has taken place and very little if any dissociation. There is remarkably good agreement between the experiments at 44,770 rpm ($\sigma \simeq 9$ cm^{-2} for mol wt 45,000) and at 31,410 rpm ($\sigma \simeq 4.4$ cm^{-2}). This agreement between experiments at different speeds was found also in the previous work and it seems with meniscus depletion experiments that more sensitivity in deciding between a mixture and an associating system is available by varying the concentration than by varying the speed. Figure 6 is a comparison of the molecular weight *vs.* concentration curves obtained for the peak tube of S1, the whole sample S1, S1 tail (Figure 1), ands ample S from a previous study (Jeffrey, 1968). There is no measurable difference between the peak tube and the whole sample and these molecular weights are in fairly good agreement with but slightly higher than those of sample S. This would reflect the presence of a bit more low molecular weight material in the latter sample which had not had so many passages through Sephadex. The tail material from the peak is obviously of different composition from S1 with respect to molecular weight, the low molecular material being present in much greater proportion.

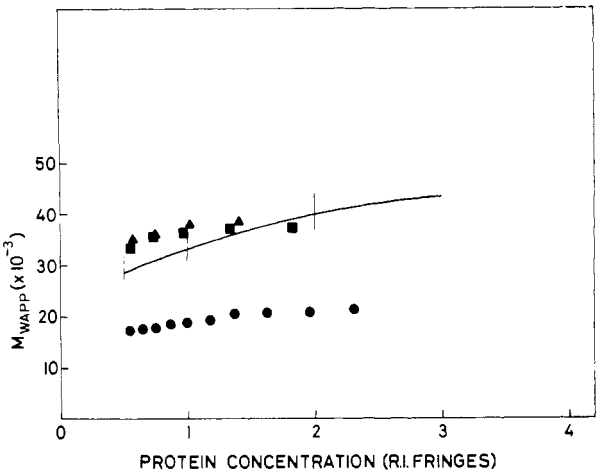

FIGURE 6: Meniscus depletion experiments with component 8 in 0.01 M sodium borate (pH 9.2) at 25°, 3-mm columns. (▲) Peak tube sample S1 (Figure 1), 0.14% 44,770 rpm. (■) Sample S1 (Figure 1), 0.10% 44,770 rpm. (●) S1, tail (Figure 1), 0.14% 44,770 rpm. The solid line is a smooth curve through the results obtained with sample S (Jeffrey, 1968) and the scatter of the points is indicated by the bars.

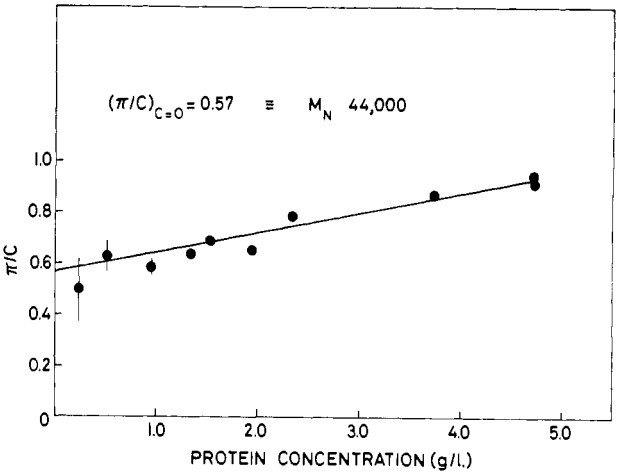

FIGURE 7: Osmotic pressure experiment with sample S2 (Figure 2) of component 8 in 0.01 M sodium borate, 25°. The points below 1.0 g/l. have large errors and are included mainly to show that the osmotic pressure does not increase at high dilutions as would be expected with an associating system.

The molecular weight curve is not very steep and a plot of $1/M_{w(app)r}$ extrapolated to zero concentration gave a molecular weight of 16,400 which is probably a fairly good estimate of the minimum molecular weight. It seems that the results of the experiments with component 8 can be explained in terms of a mixture of proteins of different molecular weights and the obvious questions are what are these molecular weights and in what proportions do they occur?

The Sephadex elution profiles (Figures 1d, 2c, and 3) seem to show that a major proportion of component 8 is a protein of single molecular weight. The decrease in measured molecular weight with concentration which is always observed in the high-speed equilibrium experiments is interpreted as being due to the fractionation of the mixture of proteins by the centrifugal field. If there is not much low molecular weight material present as the gel filtration suggests, it might be expected that the results of low-speed sedimentation equilibrium experiments which would not cause much fractionation would give a molecular weight which is a good approximation to that of most of the material. The reciprocals of the apparent weight-average molecular weights measured in low-speed sedimentation equilibrium experiments with sample S (Jeffrey, 1968) were plotted against concentration and the line through the points was extrapolated to zero concentration. The resulting molecular weight was 45,500, a value in good agreement with that of 46,000 measured in 8 M urea solutions (Jeffrey, 1968). It therefore seemed likely that the greater part of the protein in the sample had a molecular weight of about 46,000 and a determination of the molecular weight by the measurement of the osmotic pressure of sample S2 (Figure 2c) confirmed this as the extrapolated value obtained from a plot of π/c *vs.* c was 44,000 ± 3,000 (Figure 7). This experiment also confirmed the conclusion from the frontal analysis that no dissociation of component 8 occurs as the solution is diluted. Figure 7 shows that there is no tendency for the osmotic pressure to increase with decreasing concentration over the range measured although it should be pointed out that the lowest concentration it was possible to use in the osmometer was 0.025 g/100 ml which is close to 1 Rayleigh interference fringe in the sedimentation experiments. Thus the osmometry alone does not rule out a very steep dissocia ion below this concentration; however the combination of the frontal analysis and the osmotic pressure measurements makes a strong case against this possibility.

It was hoped that the mixture would only consist of proteins of two molecular weights and "two-species" plots were made to see whether this was a reasonable expectation. Two-species plots, used by Sophianopoulos and Van Holde (1964) and Roark and Yphantis (1968) in treating associating systems, are also valid for mixtures and are made by plotting the weight-average molecular weight against the reciprocal of the number-average molecular weight at the same point or the z average against the reciprocal of the weight average, etc. In ideal solutions if there are only two molecular weight species present the resulting plot is a straight line which intersects the hyperbola $M_k(r)/M_{k-1}(r) = 1$ at the two points M_1 and M_2, the required molecular weights. This method has been tested on synthetic mixtures of proteins and it has been found that when the speed is chosen to give σ for the lower molecular weight species $\simeq 5$ cm^{-2} and when the ratio of the molecular weights of the two proteins is just over 3:1, the two molecular weights can be obtained with an accuracy of 15% or better for mixtures containing 5% or more of the lower species (Jeffrey and Pont, 1969). Figure 8 shows a two-species plot for sample S (Jeffrey, 1968); sample S1 and sample S1 tail at 52,640 rpm. At this speed $\sigma \simeq 5$ cm^{-2} for mol wt 14,000. It was assumed that all protein species present had the same partial specific volume and specific refractive index increment and that the solutions were effectively ideal at the concentrations observed.

The small number of points is a consequence of the high speeds and concentrations which had to be used in order to produce sufficient fractionation to use two-species plots. The solid line in Figure 8 is a least-squares regression through the three sets of points and the correlation coefficient of this line of 0.97 shows the linearity to be significant to better than the 99.9% confidence limit. Thus it seems that these three samples of component 8 contain two molecular weight species and that

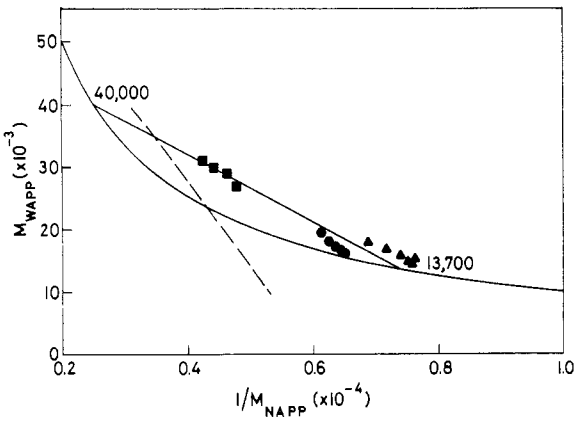

FIGURE 8: Two-species plot of molecular weights from meniscus depletion experiments with component 8 in 0.01 M sodium tetraborate, pH 9.2, 3-mm columns, 25°, 52640 rpm. (■) Sample S (Jeffrey, 1968), 0.15 g/100 ml. (●) Sample S_1 (Figure 1), 0.10 g/100 ml. (▲) Sample S_1 tail (Figure 1), 0.14 g/100 ml. The solid line is the least-squares regression line through the points. The dashed line is that for a twos-pecies plot using values of M_n based on an associating system with monomer mol w 23,000 (see text).

the two molecular weights are the same in each sample. The different position of the points along the line means that the two species are in different proportions in the three samples at the levels measured in the ultracentrifuge cell. The intersections of the line with the hyperbola give molecular weights of 40,000 and 13,700 for the two species. From the experiments with synthetic mixtures mentioned before we do not expect the molecular weights derived from such plots with a system of the present type to be within much better than 15% of the true values, and the figure of 40,000 is therefore in satisfactory agreement with that of 45,500 from low-speed sedimentation equilibrium, 46,000 in 8 M urea and 44,000 from osmotic pressure measurements. On the same basis the error in the figure for the lower molecular weight is expected to be about ±2000 and its molecular weight is taken to be 14,000 ± 2000. This is in reasonable agreement with the value of 16,400 obtained by extrapolation in an experiment at a lower speed with the S_1 tail material.

The dashed line in Figure 8 shows the two-species line given by the weight- and number-average molecular weights of sample S from a previous study (Jeffrey, 1968). In this instance because the experiments were at low speed and the conditions did not allow number-average molecular weights to be evaluated directly from the experimental results they were computed from the expression given by Adams (1965) as

$$\frac{CM_1}{\overline{M}_{napp}} = \int_0^c \frac{M_1}{\overline{M}_{wapp}} dc$$

which is applicable only to an associating system, and M_1 was taken to be 23,000. It can be seen from Figure 8 that the number-average molecular weights obtained on the basis of these assumptions are in gross disagreement with those measured in the meniscus depletion experiments. This is additional evidence against the hypothesis advanced previously to explain the sedimentation equilibrium results obtained with component 8, *i.e.*, that it is an associating system with a monomer of mol wt 23,000.

In a previous communication (Jeffrey and Pont, 1969) it was shown that in a mixture of two proteins, the proportion of a small amount of material of molecular weight one-third to one-fifth of that of the main component estimated by extrapolating M_n or M_w to the cell bottom in a high speed run of low concentration was likely to be seriously in error. In view of this no attempt has been made to utilize this method of estimating the proportions of the two proteins in the component 8 solutions. The number-average molecular weight determined by osmometry is not subject to fractionation and its lower limit of 41,000 corresponds to just under 5% of a 14,000 molecular weight species. It therefore seems as if (assuming that 14,000 and 45,500 are the only two species present) there is somewhere between 0 and 5% of the lower. To see whether such a small amount was consistent with the molecular weight distributions obtained in the high-speed sedimentation equilibrium experiments, the concentration distribution of a mixture of proteins of mol wt 14,000 and 45,500 in the proportions 5–95% was calculated from

$$C(r) = \sum_{i=1}^{n} A_i \exp(\sigma_i r^2/2) \qquad (3)$$

where $A_i = 0.5\sigma C_0(b^2 - a^2)/[\exp(\sigma b^2/2) - \exp(\sigma a^2/2)]$, C_0 is the intial concentration, and b and a are the distances of the cell bottom and meniscus, respectively, from the axis of rotation. The total initial concentration of the solution was taken as 0.14 g/100 ml, $\bar{v}$ as 0.715 ml/g, the speed as 44,770 rpm, b = 6.94 cm, and a = 6.648 cm. These values were those in a typical experiment with component 8. The resulting calculated molecular weight distribution is shown in Figure 9 together with an experiment with the peak tube from sample S1 under the same conditions, a smooth curve through the points of sample S with the scatter indicated, and the calculated monomer–trimer curve obtained previously (Jeffrey, 1968). It is immediately obvious that 5% of the 14,000 unit is more than enough to explain the results with sample S1, *i.e.*, that this sample contains something under 5% of a protein of molecular weight about 14,000 and that sample S contained very close to 5% of this material.

Discussion.

All the results presented in this paper are consistent with samples S1 and S2 of component 8 being mixtures of more than 95% of a protein of mol wt 45,000 and less than 5% of mol wt 14,000. It is considered that the rapid equilibrium proposed before (Jeffrey, 1968) to account for the sedimentation equilibrium results with sample S (and sample A) is ruled out by the results of the osmotic pressure and frontal analysis experiments unless the rather unlikely effects discussed in connection with these two techniques occur. The fact that the sedimentation equilibrium results can be fitted perfectly well on the basis of a mixture of the composition given above makes it virtually certain that this is the correct explanation. The success of the monomer–trimer and monomer–dimer–trimer hypotheses in fitting the experimental results is presumably fortuitous and must be ascribed partly to the availability of two quantities (the type of equilibrium and the value of the second virial coefficient) which can be varied, and partly to experimental error. The existence of the latter means that a cer-

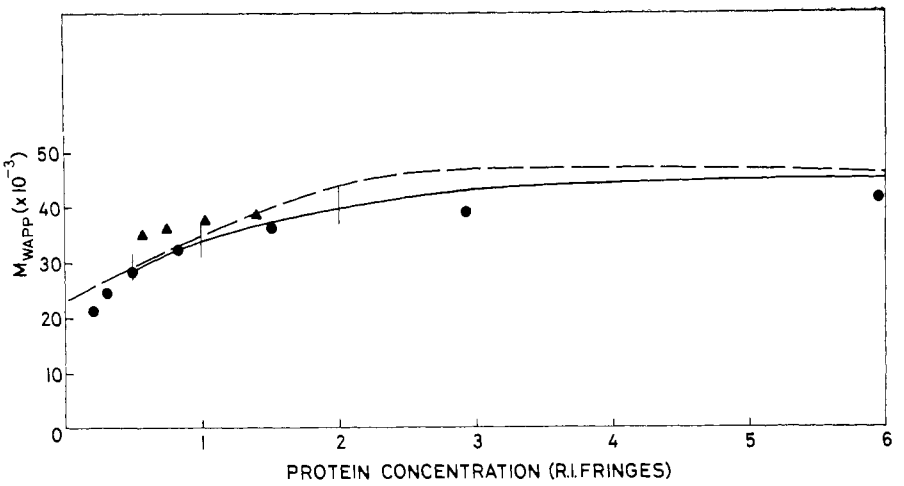

FIGURE 9: Comparison of calculated and experimental molecular weight distributions for component 8 in high-speed sedimentation equilibrium experiments at 25°, 3-mm column height. (▲) Sample S1 peak tube, 0.14% 44,770 rpm. (●) Calculated for a mixture of 95% of mol wt 45,500 + 5% of mol wt 14,000, 0.14% 44,700 rpm. The solid line is a smooth curve through the results obtained with sample S with the bars showing the scatter (Jeffrey, 1968). The dashed line is the curve obtained for the hypothetical monomer–trimer equilibrium shown in Figure 3c of Jeffrey (1968).

tain poorness of fit between calculated and experimental results is tolerated and also introduces uncertainties into the quantities used in the analysis. In particular the value chosen for the molecular weight of the monomer can have a profound effect. A value of 23,000 (obtained by extrapolation) was used in the previous analysis of the component 8 results whereas the molecular weight of the smaller species present in solution is now thought to be about 14,000. It proves to be impossible to fit the experimental results with a monomer–dimer, monomer–trimer, or monomer–dimer–trimer scheme using 14,000 as the molecular weight of the monomer. The shapes of the sedimentation velocity patterns of sample S which were stated to be consistent with the equilibria proposed (Jeffrey, 1968) are also consistent with the explanation proposed here. The lowest concentration usable in the sedimentation velocity experiments with the schlieren optical system was 0.2 g/100 ml and it looked as though partial resolution into peaks was about to occur at this concentration. The first derivative curves of the descending boundary in the frontal analysis experiments (Figure 3) are equivalent to a continuation of the sequence of sedimentation velocity (descending boundary) patterns to lower concentration and show that such resolution does not occur.

The absence of an equilibrium between the protein of mol wt 45,000 and one of lower molecular weight in 0.01 M sodium tetraborate is in agreement with experiments in 8 M urea (Jeffrey, 1968) which did not show any evidence of dissociation below a unit of mol wt 46,000. The molecular weights which have been measured for component 8 in 8 M urea are 43,000 by Yphantis' midpoint method, 45,000 by gel filtration (Thompson and O'Donnell, 1965), and 46,000 by sedimentation equilibrium (Jeffrey, 1968). The mean of these three figures is 44,700. The mean of the determinations in 0.01 M sodium tetraborate by osmotic pressure and low-speed sedimentation equilibrium which have been described in this paper is 44,800, or if the two-species value is included, 43,200. It is therefore considered that the molecular weight of more than 95% of the lower sulfur wool protein denoted component 8 and prepared as described in this paper is very close to 45,000. There is little doubt that component 8 is heterogeneous chemically (Thompson and O'Donnell, 1967; Frater, 1968) and the fact that more than 95% of it appears to have the same molecular weight lends some support to the view that component 8 consists of a family of related proteins which differ in amino acid sequence in noncritical regions of the peptide chain (Thompson and O'Donnell, 1967).

The identity of the small percentage of protein of mol wt 14,000 ± 2000 which is present even in the most highly fractionated sample of component 8 studied is not known. The presence of this material was suspected before (Jeffrey, 1968) but no estimate of its molecular weight was then available for use in performing the calculation whose result is shown in Figure 9, and its potentially enormous effect on the molecular weight distribution in high-speed sedimentation equilibrium experiments was therefore not appreciated. Amino acid analyses of the tails of peaks from gel filtration on Sephadex suggest that this region contains high-sulfur and high-glycine–tyrosine proteins and these may be the low molecular weight material in the component 8 solutions. The molecular weight of a high-glycine–tyrosine protein from merino wool has been reported to be in the range 9,000–17,000 (Zahn and Biela, 1968) while several of the high-sulfur wool proteins are in the range 11,000–17,000 (Joubert *et al.* 1967) and high-sulfur proteins are known to be present as contaminants of component 8 (Frater, 1968).

There is some disagreement in the literature (Crewther *et al.*, 1965) about the molecular weight of SCMKA, of which component 8 forms a large proportion (Thompson and O'Donnell, 1964, 1965), and it is possible that at least part of this could be resolved if the presence of some low molecular weight material in component 8 were taken into account. However further speculation seems premature until the planned studies of component 7, the other major constituent of SCMKA, are completed.

Acknowledgments

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References

Adams, E. T., Jr. (1965), *Biochemistry 4*, 1646.
Casassa, E. F., and Eisenberg, H. (1964), *Advan. Protein Chem. 19*, 287.
Crewther, W. G., Fraser, R. D. B., Lennox, F. G., and Lindley, H. (1965), *Advan. Protein Chem. 20*, 191.
Frater, R. (1968), *Australian J. Biol. Sci. 21*, 815.
Harrap, B. S., and Woods, E. F. (1958), *Australian J. Chem. 11*, 581.
Harrap, B. S., and Woods, E. F. (1964), *Biochem. J. 92*, 8, 19.
Jeffrey, P. D. (1968), *Biochemistry 7*, 3345, 3352.
Jeffrey, P. D., and Pont, M. J. (1969), *Biochemistry 8*, 4597.
Joubert, F. J., De Jager, P. J., and Swart, L. S. (1967), *in* Symposium on Fibrous Proteins, Crewther, W. A., Ed., Sydney, Butterworths, p 343.
Roark, D. E., and Yphantis, D. A. (1968), *Biophys. J. 8*, A99.
Ronalds, J. A., and Winzor, D. J. (1969), *Arch. Biochem. Biophys. 129*, 94.
Sophianopoulos, A. J., and Van Holde, K. E. (1964), *J. Biol. Chem. 239*, 2516.
Tanford, C. (1963), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 291.
Thompson, E. O. P., and O'Donnell, I. J. (1965), *Australian J. Biol. Sci. 18*, 1207.
Thompson, E. O. P., and O'Donnell, I. J. (1967), *Australian J. Biol. Sci. 20*, 1001.
Winzor, D. J., and Nichol, L. W. (1965), *Biochim. Biophys. Acta 104*, 1.
Winzor, D. J., and Scheraga, H. A. (1963), *Biochemistry 2*, 1263.
Winzor, D. J., and Scheraga, H. A. (1964), *J. Phys. Chem. 68*, 338.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zahn, H., and Biela, M. (1968), *European J. Biochem. 5*, 567.